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<b>(21) International Application Number:</b> PCT/US90/00182 <b>(22) International Filing Date:</b> 9 January 1990 (09.01.90)  <b>(30) Priority data:</b> 295,637 10 January 1989 (10.01.89) US  <b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US).  <b>(72) Inventor:</b> McLAUGHLIN, Larry, W. ; 33 Beech Street, Needham, MA 02194 (US).  <b>(74) Agent:</b> SCOTT, Anthony, C.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS  <b>(57) Abstract</b> <p>The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar (<math>10^{-15}</math> mol) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.</p>		

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LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS

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FIELD OF THE INVENTION

The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes such as spin labels and drug analogues into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar ( $10^{-15}$  moles) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can also be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.

BACKGROUND OF THE INVENTION

The determination of the presence of nucleic acid fragments has typically relied on the use of radioisotopic labeling techniques. The enormous utility of these techniques has largely been a function of the high sensitivity associated with their detection. Such

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1 sensitivity has allowed the location of quantities of  
material in amounts in the low femtomolar range ( $10^{-15}$   
moles). However, the use of radioisotopes is rendered less  
than ideal by the associated problems of safety and disposal.

5 Fluorescent rather than radioisotopic labeling  
procedures are an attractive option which avoids these  
liabilities, but fluorescent labeling procedures have  
previously been compromised by their greatly reduced  
sensitivity. Fluorescent dyes as well as spin labels are  
10 also useful in many aspects of biophysics since the  
properties of a given marker can vary substantially with  
changes in the immediate microenvironment. Such probes can  
be useful for the study of structure, conformation and  
dynamics in biopolymers providing that they can easily be  
15 placed at specific locations within the desired  
macromolecule.

In order for fluorescent labeling procedures to  
compete effectively with and replace radioisotopic labeling  
techniques for the detection of macromolecules during various  
biochemical assays, the fluorescent labeling must result in  
20 high detection sensitivity, rapid and simple procedures for  
the introduction of the fluorescent marker to the  
macromolecule of interest must be available, and the results  
must be reproducible. By meeting these criteria and with the  
additional advantage of reduced health hazards, fluorescent  
25 labeling techniques could then replace the use of  
radioisotopes in a number of biochemical assays.

Intercalative dyes such as ethidium bromide  
generally meet these criteria and in many cases have  
completely replaced radioisotopic labeling procedures for the  
30 detection of double stranded DNA. However, a number of  
assays, including DNA sequencing and hybridization

1 techniques, cannot benefit from intercalative fluorescent labeling. These procedures require that the fluorescent marker be covalently bound to the nucleic acid, and the intercalative dye is unable to meet this requirement.

5 All prior studies for the covalent attachment of fluorescent markers to nucleic acids, until the present invention, suffered from at least one of two disadvantages. First, attachment of only a single label to the nucleic acid (usually at one of the termini) severely compromised its detection. Secondly, although multiple labeling techniques  
10 can enhance detection sensitivity, they have generally required the time-consuming synthesis of a modified nucleoside derivative containing a fluorophore or one which can be modified with a fluorophore. In addition to fluorophores, the use of biotin as a non-radioactive labeling  
15 technique has also been considered.

The use of single labels, usually at the terminus of the nucleic acid fragment, is the conventional state of the art primarily because it is chemically and enzymatically easier to exploit modification reactions at a nucleic acid  
20 terminus rather than at a specific point in the internal regions of the sequence. Additionally, the placement of the marker at one of these termini also removes the marker from the "site of action" when monitoring protein binding or any process where an essentially native DNA sequence is required.  
25 It has commonly been difficult to detect fragments containing a single fluorescent marker with the high sensitivity available with a radioisotopic label. Although problematic, labeling with a single fluorophore has been accomplished using both chemical and enzymatic techniques. DNA sequencing  
30 has been attempted using such labeling techniques but requires sophisticated electronic detection, and then only has evidenced limited success.

Several methods have been reported for the  
1 incorporation of multiple labels into nucleic acids. Most of  
these rely on an enzymatic polymerization reaction in order  
to introduce a modified nucleoside carrying the desired label  
or one which can be easily modified with the fluorescent  
5 marker at numerous positions. Base-specific reactions have  
also been employed, such as modification of guanine residues  
with N-acetoxy-2-acetylaminofluorene followed by detection  
with tetramethylrhodamine-labeled antibodies raised against  
the modifying reagent. Multiple labeling techniques have  
10 commonly resulted in enhanced detection sensitivity with  
respect to single labels and have been reasonably  
reproducible. However, these techniques have previously not  
been simple or rapid to employ. The modified nucleoside has  
previously only been obtained by time-consuming chemical  
15 syntheses.

Another prior approach involves the use of biotin  
labeling. While biotin itself is not a fluorescent  
chromophore, biotin labeling when combined with  
immunochemical, histochemical or affinity detection systems  
20 provides another alternative to radioisotopic labeling of  
nucleic acids. Biotin-labeled nucleic acids have been used  
in hybridization studies, gene mapping studies employing  
electron microscopy and gene enrichment in cesium chloride  
gradients. Biotin labeling has been typically approached in  
25 conceptually the same manner as fluorescent labeling  
techniques in which either a single label at the nucleic acid  
terminus or multiple labels requiring the synthesis of a  
biotin labeled dNTP derivative are employed. Generally, each  
of the existing techniques suffers from the requirements of  
30 arduous chemical synthesis and/or limited detectability.

Conventional techniques when applied to DNA sequencing procedures add additional complications since the DNA fragments prepared during sequencing techniques must be resolved by electrophoresis in a polyacrylamide gel matrix. Since electrophoresis procedures resolve nucleic acid fragments on the basis of size (or molecular weight), the addition of one or more fluorescent labels to the fragments prior to electrophoresis results in anomalous migration of the DNA within the gel and undue complications in the analysis of the sequence. The most desirable procedure for employing fluorescent labeling techniques in DNA sequencing and hybridization procedures would involve the incorporation of multiple labels into the nucleic acid or hybridization probe (to enhance detection sensitivity), before or after electrophoretic resolution of such fragments or before or after hybridization of the probe onto a nitrocellulose membrane ("pre-assay" or "post-assay" labeling). Multiple covalent labeling of nucleic acids with fluorophores in a "post-assay" manner has not been previously contemplated or described.

#### SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide an improved method for labeling nucleic acids.

Another object of this invention is to provide an improved method of fluorescently labeling nucleic acids.

A further object of the present invention is to provide new probes for use in DNA labeling and related techniques.

A still further object of this invention is to provide a new detection product which constitutes a phosphorothiolate diester covalently complexed with a nucleotidic residue, and which is also complexed with a detectable marker.

Another object of this invention is to provide  
1 multiple sites, i.e., internally within the macromolecule,  
for the attachment of fluorophores and other markers and/or  
probes to the nucleic acid thereby enabling multiple labeling  
techniques.

5 A further object of the present invention is to  
selectively introduce fluorescent markers and other markers  
and probes at specifically desired sites of the  
macromolecule. These markers or reporter groups include  
fluorophores, biotin, spin labels, drugs or their analogues,  
10 hydrolytic reagents, chiral metal complexes and the like.

Another object of this invention is to selectively  
introduce fluorescent markers and other probes after the  
molecule of interest has been treated with any one of various  
desired biochemical assays, i.e., in a "post-assay"  
15 procedure.

Still another object of this invention is to  
selectively introduce fluorescent markers and other probes  
before the molecule of interest has been treated with any one  
of various desired biochemical assays, i.e., in a  
20 "pre-assay" procedure.

Yet another object of the present invention is to  
provide an improved process for DNA sequencing, DNA  
hybridization techniques and DNA diagnostics and DNA  
therapeutics.

25 A still further other object of this invention is  
to provide a new detection procedure which eliminates the use  
of radioisotopes and the disadvantages associated with such  
conventional methods.

30 These and other objects of the present invention  
are achieved by providing a protocol which permits the  
covalent introduction of single or multiple markers,  
particularly fluorescent markers, and other probes into DNA  
fragments and oligodeoxynucleotides at selective sites. More

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specifically, according to the present invention, nucleic  
1 acids are labeled with markers such that, e.g., the  
fluorescent marker or any other type of probe can be placed  
into a specific location in the nucleic acid. By the  
technique of the present invention, various sites for the  
5 attachment of the desired probes or markers are generated by  
employing phosphorothioate diesters in place of native  
phosphodiester which are chemically or enzymatically  
introduced at the desired site within a nucleic acid and  
subsequently marked with the desired reporter group. The  
10 present methodology not only permits multiple labeling and  
high sensitivity in a simple technique in the absence of  
sophisticated detection devices, but also permits the  
introduction of a particular probe or marker after  
conventional biochemical assays, i.e., "post-assay." The  
15 advantages of the novel detection products of this invention  
also allow the labeling of DNA fragments in conventional DNA  
sequencing or hybridization assays. Such assays further  
permit a host of therapeutic procedures where a DNA  
hybridization probe with attached phosphorothioate diester(s)  
20 is employed in vivo or in vitro to locate a sequence within  
genomic DNA and which is subsequently reacted with, e.g., a  
label for detection or identification, a reactive molecule  
for degradation, or other toxic therapeutic agents. The  
novel product also allows study of the structure and dynamics  
25 of nucleic acids as well as protein-nucleic acid complexes.  
The novel product of the present invention includes a  
nucleotidic residue covalently complexed with a  
phosphorothioate diester and further complexed to a marker  
enabling detection of the product.

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BRIEF DESCRIPTION OF THE DRAWINGS

1           Fig. 1 sets forth the structure of the  
phosphorothioate triester composed of the nucleotidic residue  
and phosphorothioate diester complexed with the bimane label  
(bimane-Tp(S)T triester).

5           Fig. 2 is a graphic illustration of the stability  
of the bimane-Tp(S)T triester at ambient temperature measured  
during a total time period of 20 hours at pH values between  
3-11.

10           Fig. 3 is a graphic depiction of an HPLC analysis  
of the reaction mixture containing the octamer d[GC(s)CCGGGC]  
(0.3 mM) and monobromobimane (3.0 mM) after reaction for  
5 hours at ambient temperature.

15           Fig. 4 is a photographic reproduction of a  
polyacrylamide gel (6%) illustrating "post-assay" labeling of  
DNA fragments with monobromobimane.

            Fig. 4(A) represents an HpaII restriction  
endonuclease digest of an M13mp18 DNA template, which has  
been elongated with DNA polymerase I (E. coli) using dNTPs  
and then treated with the endonuclease.

20           Fig. 4(B) represents an AvaI restriction  
endonuclease digest of an M13mp19 DNA template, which was  
elongated with DNA polymerase I (E. coli) using dNTPs and  
then treated with endonuclease.

25           Fig. 5 represents phosphorothioate triester  
oligodeoxynucleotides carrying (a) a PROXYL spin label. (b)  
a derivative of the dihydropyrroloindole subunit of CC-1065,  
(c) a sulfonamide-linked dansyl fluorophore, and (d) an  
N-linked dansyl fluorophore.

DETAILED DESCRIPTION OF THE INVENTION

30           The present invention contemplates the selective  
labeling of nucleic acids with fluorescent molecules and  
other probes such as, for example, biotin, which are useful  
in DNA sequencing and DNA hybridization assays. The present

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invention also contemplates other probes such as, for  
1 example, spin labels which are useful in the analysis of  
nucleic acid structure and dynamics. The convenient labeling  
methodology of this invention further permits a broad range  
of DNA therapeutic and diagnostic procedures and is  
5 particularly characterized by the selective covalent  
introduction of single or multiple markers and probes into  
DNA fragments and oligodeoxynucleotides. The novel detection  
product of this invention is characterized by a nucleotidic  
residue covalently complexed with a phosphorothioate diester  
10 which is mutually covalently complexed with a selected  
marker. The probe is selectively introduced into a single  
site of choice or into multiple sites as desired.

The present invention preferably employs a  
phosphorothioate diester [for example, Tp(s)T,  
15 phosphorothioate diester derivative of TpT (thymidyl(3'→5')  
thymidine)] which is selectively incorporated into a DNA  
fragment or oligodeoxynucleotide at any and each nucleotide  
residue desired.

Specifically, the probe of the present invention, a  
20 phosphorothioate diester derivative, is prepared by  
introducing the phosphorothioate diester into the nucleic  
acid fragment either enzymatically, e.g., according to the  
method of Potter and Eckstein (Potter, B. and Eckstein, F.,  
J. Biol. Chem., 259: 14243-14248, 1984), or chemically,  
25 e.g., according to the method of Connolly, et al. (Connolly,  
et al., Biochemistry, 23: 3443-3453, 1982).

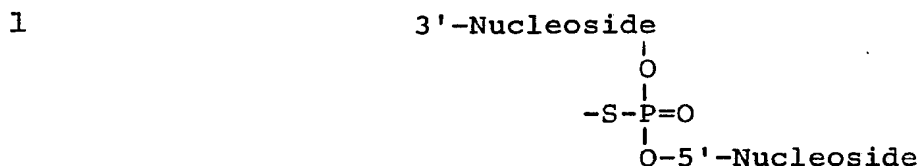
The enzymatic technique of Potter and Eckstein  
employs the desired dNTP  $\alpha$  S 2'-deoxynucleoside-5'-0-  
(1-thiotriphosphate), a suitable enzyme with polymerizing  
30 characteristics such as DNA polymerase or reverse  
transcriptase, a DNA template and a primer. The enzyme  
employed, uses dNTP S as a substrate to synthesize nucleic  
acids of varying chain length, and upon enzymatic reaction, a

1 phosphorothioate diester is incorporated between two  
nucleoside residues, along with the concurrent liberation of  
pyrophosphate.

5 The phosphorothioate diester may be introduced  
chemically into the nucleic acid by the method of Connolly,  
et al. (or Stec, et al., J. Am. Chem. Soc., 106: 6077-6079).  
This is generally a three step procedure. First, a phosphite  
triesters (nucleoside phosphite triester) is formed by  
10 reacting a nucleoside phosphoramidite in the presence of a  
weak acid such as tetrazole. Second, the phosphite triester  
is oxidized in the presence of elemental sulfur ( $S_8$ ),  $CS_2$  and  
lutidine, to form a phosphorothioate triester complex.  
Third, in the presence of a base such as ammonia, the  
phosphorothioate triester is hydrolyzed to the desired  
phosphorothioate diester.

15 The selective introduction of the phosphorothioate  
diester derivative into the DNA fragment or  
oligodeoxynucleotide, is determined by the choice of  
oxidation procedures at any given position. As explained  
above, the phosphorothioate diester is obtained by oxidation  
20 in the presence of  $S_8$ ,  $CS_2$  and lutidine. The native  
phosphate diester is obtained by oxidation of the phosphite  
triesters with a mixture of  $I_2$ , THF (tetrahydrofuran),  $H_2O$  and  
lutidine followed by hydrolysis of the triester to yield a  
phosphate diester. The appropriate choice of either set of  
25 conditions allows the placement of the phosphorothioate  
diester in the desired position with respect to the native  
phosphate diester. This technique allows for selective  
reactivity at a specific nucleotidyl site, and avoids  
nonspecific reaction with other functional groups available  
30 in the nucleic acid.

The complex formed is described below:



5 (Internucleotidic Phosphorothioate Diester)

The phosphorothioate diester can subsequently be alkylated with fluorescent molecules or other probes such as, for example, biotin. In this procedure, the complex which results is referred to as a "phosphorothioate triester" (which comprises an internucleotidic residue, a phosphorothioate diester and a detectable marker). The means by which this procedure occurs, e.g., alkylation, refers to the displacement of the functional group (such as the bromine in monobromobimane) and the formation of a sulfur-carbon bond between the fluorescent marker and the phosphorothioate diester.

For purposes of fluorescent labeling techniques herein contemplated, various fluorophores can be employed, for example, monobromobimane (MBB), bromomethylcoumarin, as well as a variety of chromophores carrying bromoacetamides, iodoacetamides, aziridinosulfonamides or  $\gamma$ -bromo- $\alpha,\beta$ -unsaturated carbonyls; monobromobimane is preferred.

One of the most surprising advantages of this invention is that the present methodology permits the introduction of fluorescent dyes or other probes in a "post-assay" procedure. By "post-assay" procedure is meant, generally, that the phosphorothioate diester-containing DNA is used in the assay of choice, for example, in polyacrylamide gel electrophoresis, and the fluorescent molecule or other marker or probe can be introduced at a later time, for example, while the nucleic acid is embedded

1 in the polyacrylamide gel matrix. The assay procedures  
contemplated by the present invention in this context  
include, for example, gel electrophoresis, Southern  
hybridization, and DNA sequencing techniques such as are  
described by Sanger, et al. (Sanger, et al., Proc. Natl.  
5 Acad. Sci., 74: 5436-5467, 1977).

Gel electrophoresis as used here is typically  
performed by running DNA samples down specific lanes in a gel  
(e.g., a polyacrylamide gel or agarose gel), under controlled  
current and temperature conditions for a short period of  
10 time. This procedure leaves the DNA embedded in the gel  
matrix.

Southern hybridization involves the use of a  
blotting membrane to remove the fractionated nucleic acid  
from the gel and allows for hybridization of labeled probes  
15 to the nucleic acid on the surface of the blotting membrane.  
Radioisotopic labeling ( $^{32}\text{P}$ ) has been commonly employed for  
the detection of nucleic acids resolved by electrophoresis or  
after hybridization techniques.

Sanger DNA sequencing (also known as "dideoxy  
20 sequencing") has previously been done using  $^{35}\text{S}$  labeling.  
This typically involves two steps. The labeling reaction is  
initiated after annealing of the primer to the template. A  
low concentration of dTTP, dGTP, dCTP and  $\alpha$ -[ $^{35}\text{S}$ ]dATP is  
employed in order to elongate the primer and incorporate some  
25 radioisotope. The second step involves adding the  
termination mixture, which is a higher concentration of all  
four dNTP derivatives plus one of the dideoxy derivatives  
(ddNTP).

Post-assay fluorescent labeling techniques as  
30 described herein permit the introduction of multiple  
fluorescent molecules or other appropriate markers into the

1 nucleic acid, e.g., after electrophoresis and "post-assay"  
labeling of detecting oligodeoxynucleotides and DNA fragments  
can be detected on the basis of, e.g., fluorescence, with  
high sensitivity.

5 Detection of fluorescent markers can be achieved by  
use of e.g., a standard long-wavelength ultraviolet  
transilluminator, to view the DNA in the gel.

10 The labeling procedure is particularly useful in  
conventional enzymatic procedures for the sequencing of DNA.  
Instead of radioisotopic labeling as described in the Sanger  
sequencing technique the four dNTP $\alpha$ S derivatives used in the  
sequencing reaction can be substituted such that the DNA  
fragments produced will contain phosphorothioate diesters at  
all internucleotidic positions which can allow multiple  
labeling and ultimately allow reading of large and small DNA  
15 fragments. The labeling procedure is also applicable to site  
specific identification of nucleotides by introducing at  
least one phosphorothioate diester selectively into an  
internucleotidic residue or DNA fragment or oligodeoxy-  
nucleotide, labeling said phosphorothioate diester with a  
20 marker and detecting said marker.

The aforesdescribed labeling technique can also be  
applicable to hybridization studies using, e.g.,  
membrane-bound nucleic acids.

25 A fluorescently labeled cloned DNA probe can be  
used to localize specific nucleic acid sequences in mixtures  
of DNA restriction fragments fractionated by gel  
electrophoresis. A replica of the gel is made by  
transferring all of the fractionated DNA fragments to a sheet  
of nitrocellulose paper or similar membrane (the "blotting  
30 membrane") by diffusion or electrophoresis. The  
hybridization probe can be labeled before or after the

1 hybridization assay occurs. The locations of the fragments  
that hybridize to fluorescently labeled DNA probes are then  
identified by their fluorescence. Similarly, nitrocellulose  
paper replicas can be made of crowded colonies of bacteria  
growing on an agar surface so that hybridization of the paper  
5 with a specific labeled probe can be used to identify the few  
cells carrying a newly cloned specific DNA fragment.

The labeling and detection techniques herein  
discussed, can also surprisingly be easily employed in DNA  
diagnostics and DNA therapy. The present advantage, relative  
10 to art recognized techniques, is particularly manifest in  
that the presence of the phosphorothioate diester does not  
effectively alter the biophysical nature of the DNA and yet  
selectively introduces a nucleophilic site which is readily  
modified and exploited for diagnostic and therapeutic  
15 purposes. For example, the phosphorothioate diester can be  
introduced into the DNA and subsequently hybridized to a gene  
of interest in vitro or in vivo, and then followed by  
specific introduction of a probe to that gene. The probe to  
the particular gene can then be used to discover the location  
20 of the gene. This leads to detection of the presence or  
absence of the gene under diagnostic investigation. The  
probe can then be used in DNA therapeutics to inactivate or  
destroy that particular gene or if necessary, to activate  
that gene. For example, diagnosing genetic disorders and  
25 direction of drug delivery (e.g., anticancer or antiviral  
drugs).

Another surprising advantage of the present  
invention is that the DNA-containing phosphorothioate diester  
is largely resistant to nucleases and therefore is very  
30 stable when introduced into complex biological systems found  
in vitro and in vivo.



1       The present invention can be used in spectroscopic  
analysis (e.g., Nuclear Magnetic Resonance studies, and in  
particular, the Nuclear Overhauser Enhancement [NOE]) to  
measure distances within nucleic acids by use of probes which  
can label specific phosphorothioate diesters.

5       The present invention can also be applied to  
Electron Spin Resonance studies, which previously relied upon  
the use of non-specific labeling. The simple and rapid  
procedures described here will allow the preparation and  
study of nucleic acid fragments containing spin labels,  
10       attached at well-characterized locations. The procedure  
described herein can also be used for the specific attachment  
of hydrolytic reagents (e.g., ferric ion complexes),  
intercalators and proteins to nucleic acids.

15       Additionally, the present invention can also be  
used to probe the structure of DNA fragments or  
oligodeoxynucleotides by using chiral metal complexes (e.g.,  
the  $\Lambda$ -isomer or  $\Delta$ -isomer of tris-(4,7-diphenylphenan-  
throlin) cobalt (III)) as the one marker of choice to be  
attached to the phosphorothioate diester.

20       In order to use the phosphorothioate diester  
effectively in a procedure for detecting nucleic acids, it is  
advantageous to assess the stability, particularly with  
respect to pH, of the labeled phosphorothioate diester-  
fluorescent marker product. An HPLC analysis can be used  
25       employing a reversed phase column. This assays the stability  
of the labeled phosphorothioate derivative (triester) over a  
broad pH range during an incubation period at ambient  
temperature.

30       In another aspect of the present invention, high  
detection sensitivity of fluorescent labeled nucleic acids  
can be facilitated by the introduction of multiple

1 fluorescent markers to a corresponding multiple number of  
phosphorothioate diesters earlier introduced at the selected  
internucleotidic sites; the labeling reaction must occur at  
adjacent phosphorothioate diesters such that, to achieve  
maximum sensitivity, a nucleic acid fragment carries a  
5 fluorophore at each and every internucleotidic phosphorus  
residue. Surprisingly, experimentation indicates that there  
is no steric hindrance or other difficulty in placing  
fluorescent labels on adjacent phosphorothioate diesters,  
thus permitting maximization of this technique.

10 As earlier discussed, "post-assay" labeling  
procedures are useful for a variety of biochemical assays;  
one of the most important specific applications involves the  
detection of nucleic acids resolved by gel electrophoresis  
techniques. One "post-assay" labeling procedure, for  
15 example, can be accomplished using short oligodeoxynucleotide  
fragments resolved by a given assay (e.g., gel  
electrophoresis) and then soaking the gel containing the  
small nucleic acid fragment with a solution which contains  
the fluorescent marker of choice. Small fragments with  
20 several labeled phosphorothioate diesters are quantitatively  
compared with the fluorescence exhibited by a nucleic acid  
fragment with a single fluorophore. There is a concomitant  
increase in detection sensitivity with an increase in the  
number of labeled phosphorus residues.

25 Longer DNA fragments containing phosphorothioate  
diesters can be prepared by enzymatic synthesis when the  
normal dNTP substrates are replaced by  $\alpha$ -thio derivatives  
(dNTP  $\alpha$ S). In order to generate fragments of defined length,  
an oligodeoxynucleotide primer can be extended using a  
30 template (e.g., M13mp18 or M13mp19 or other single-stranded  
DNA) and then the resulting material can be hydrolyzed with

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1 an appropriate restriction endonuclease. The amount of DNA  
fragment which can be visualized is approximated based upon  
the maximum amount of template present in the reaction  
mixture or as the result of internal standardization via  
radioisotopic labeling. The variety of bands produced can be  
5 visualized by "post-assay" fluorescent labeling procedures.  
The results show a further increase in sensitivity relative  
to the increased sensitivity in small nucleic acid fragments.

Various fluorophores are available and many can be  
employed in the present process. Any fluorophore can be  
10 utilized for the "post-assay" fluorescent labeling procedures  
contemplated by the present invention which reasonably  
possess the following properties: high quantum yield;  
solubility in aqueous (or largely aqueous) solutions;  
relatively small size to allow diffusion through the gel  
15 matrix; high fluorescence only after reaction with a sulfur  
residue; and removal of the excitation maximum from the  
absorbance maximum of the nucleic acids. One preferred  
fluorophore which meets these criteria is monobromobimane.  
Other fluorophores of choice can include, for example,  
20 bromomethylcoumarin, or fluorophores carrying bromo- or  
iodoacetamides, or aziridinosulfonamides. The fluorophores  
of choice have the ability to alkylate the phosphorothioate  
diester. The phosphorothioate diester is more nucleophilic  
than any other site on the nucleic acid and results in  
25 formation of a stable phosphorothioate triester when labeled  
with the fluorophore of choice.

In particular, two widespread assays which can be  
employed in conjunction with the "post-assay" fluorescent  
labeling of this invention are DNA sequencing using, e.g.,  
30 the Sanger dideoxy method and DNA hybridization (using e.g.,  
the Southern technique).

# 1. DNA Sequencing

1           Post-assay labeling is most amenable to enzymatic  
dideoxy sequencing procedures. This approach incorporates  
phosphorothioate diesters in place of native phosphate  
diesters in the DNA fragments generated. After gel  
5   electrophoresis, multiple fluorophores, such as MBB, can be  
attached to the DNA via alkylation of the sulfur residue of  
the phosphorothioate diesters.

          Current technology of Sanger sequencing utilizes  
the dNTP derivatives. The Sanger sequencing technique  
10   commonly utilizes a single  $\alpha$ -[S<sup>35</sup>]dNTP derivative to  
introduce the radioactive label. However, by using all four  
dNTP  $\alpha$ S derivatives in the present invention, DNA fragments  
can be generated by this technique which can contain hundreds  
of phosphorothioate diesters. The "post-assay" labeling of  
15   this invention can be directly applied to the detection of  
these fragments.

          The "post-assay" fluorescent labeling technique  
provides the sensitivity necessary to visualize DNA  
sequencing ladders in the absence of radioisotopes. The  
20   technique as described here employs all four dNTP  $\alpha$ S  
derivatives plus one of the dideoxy derivatives (ddNTP) in  
the elongation and then termination of the DNA primer.  
Sequencing ladders can be generated with dNTP  $\alpha$ S substrates  
in the like manner to the methodology with dNTP derivatives.

25           It is then desirable to vary the elongation and  
termination conditions such that in the initial fluorescence  
labeling the amount of DNA in each band may be varied. Then  
the amount of DNA that appears in the bands can be maximized,  
e.g., ranging from approximately 300 to 500 base pairs.  
30   Fragments of this size can be resolved, and 300 to 500  
fluorophores or other types of markers can be incorporated

1 into such fragments. The distribution of the fragments can  
be altered by changing the relative ratios of the  
dideoxynucleotide/deoxynucleotides triphosphates.

5 A ddNTP/dNTP $\alpha$ S ratio of about 1:10 may be used to  
obtain a distribution of small and large fragments. A  
decrease in this ratio is effected to allow for more  
efficient polymerization in a stepwise manner to as low as  
about 1:500 in order to shift the distribution to longer  
fragments.

10 The use of  $\alpha$ -[<sup>35</sup>S]dATP as a method for introducing  
the radioisotopic label has been reported and is commonly  
employed. Dideoxy sequencing using <sup>35</sup>S labeling typically  
involves two steps. After annealing of the primer to the  
template the labeling reaction is initiated. A low  
concentration of dTTP, dGTP, dCTP and  $\alpha$ -[<sup>35</sup>S]dATP is  
15 employed in order to elongate the primer and incorporate some  
radioisotope. The second step involves adding the  
termination mixture which is a higher concentration of all  
four dNTP derivatives plus one of the dideoxy derivatives  
(ddNTP). It is a simple procedure to then substitute the  
20 four dNTP $\alpha$ S derivatives in both reactions (actually there is  
only one reaction since no radioisotopic labeling is  
involved) such that the DNA fragments produced will contain  
phosphorothioate diesters at all internucleotidic positions.

25 For internal standardization, radioisotopic  
labeling can be used in combination with fluorescent markers  
to monitor the limits of detection sensitivity. To obtain  
fragments which have been labeled to a known specific  
activity a "minus-dCTP" labeling reaction is employed. This  
uses a primer and template of known sequence, for example, of  
30 the following sequences:

1 M13mp18 3'...CAAAGGGTCAGTGCTGCAACATTTTGCT...5'  
primer 5'-GTTTTCCCAGTCACGAC-3'.

The labeling reaction can now be performed with low concentration of the dTTP  $\alpha$ S, dGTP  $\alpha$ S and  $\alpha$ -[<sup>35</sup>S]dATP. The elongation of the primer proceeds until the first dG present in the template and then terminates resulting in the following sequence containing four <sup>35</sup>S labels:

M13mp18 3'...CAAAGGGTCAGTGCTGCAACATTTTGCT...5'  
elongated primer 5'-GTTTTCCCAGTCACGACGTTCTAAAA-3'

\*\*\*\*

10 The termination reaction uses all four dNTP  $\alpha$ S derivatives at concentrations some two orders of magnitude higher than the labeling reaction such that any remaining radioactive  $\alpha$ -[<sup>35</sup>S]dATP is diluted and the quantity available for incorporation becomes insignificant. The amount of material present (based upon the known specific activity of the  $\alpha$ -[<sup>35</sup>S]dATP) in a given band can now be easily determined by excising the band, lyophilizing the gel and determining the radioactivity present by scintillation counting. By adjusting the concentrations of the template and primer as well as the ratio of the ddNTP to dNTP  $\alpha$ S, the amount of DNA present in a given fragment can be altered. In addition, distribution of fragments can be shifted to those of higher or lower molecular weight. Optimization of detection can allow "reading" of smaller fragments (smaller than 300 nucleotide residues). DNA sequencing in the absence of radioisotopes can then be effectuated by detecting the hundreds of labeled, e.g., biplane-labeled phosphorothioate triesters by utilization of single or sophisticated electronic techniques.

30

35

## 2. DNA Hybridization

1           In another embodiment of the present invention, the  
post-assay fluorescent labeling technique can also be applied  
to hybridization studies using nucleic acids. The stability  
of a native DNA duplex is first tested against nucleic acid  
5 containing a number of phosphorothioate diesters and the  
effect of this stability when the phosphorothioate diesters  
are alkylated by a fluorophore is determined. For example,  
the results for the detection of a 21-mer fragment containing  
20 phosphorothioate diesters shows that in the absence of  
10 electronic instrumentation it can readily be detected  
visually. Nucleic acids with one label can be detected and  
detection of single nucleotides can be facilitated. Such  
visibility is increased proportionately with the proportionate  
number of markers.

15           A 21-mer fragment is one example of a small  
hybridization probe which can be used to detect nucleic acid  
sequences. This is utilized in the following manner: DNA  
fragments or oligodeoxynucleotides of reproducible size are  
generated by selective chemical means, such as by a  
20 restriction endonuclease enzyme. These nucleic acids are  
resolved by a biochemical assay such as polyacrylamide or  
agarose gel electrophoresis. The nucleic acid resolved in  
this manner is then transferred to a blotting membrane, e.g.,  
nitrocellulose membrane and the DNA probe is hybridized to  
25 the nucleic acid. Although the DNA probe at this point has  
the phosphorothioate diester or diesters incorporated into  
it, the marker of choice, e.g., a fluorescent marker, may be  
introduced before or after the hybridization assay.  
Following these steps, the marker can be detected using  
30 simple or sophisticated detection techniques.

One of the primary differences between "post-assay" fluorescent labeling within a gel matrix and labeling on a blotting membrane is that the latter occurs primarily on the surface of the membrane and not within a three dimensional matrix. With such surface phenomena it is possible to also use biotin labeled hybridization probes and detection with fluorescent protein complexes which could not be used for labels embedded in a gel matrix (the proteins involved are of large molecular weight and would not readily diffuse through the pores of the gel matrix). The phosphorothioate diester can be employed to allow efficient multiple (and specific) labeling with a biotin derivative. For example, the bromoacetamido group can be used to modify the phosphorothioate diester. A biotin derivative containing this functional group can be prepared quite simply by techniques available to one of ordinary skill in the art. Biotin labeling in this manner is considered an effective method for detecting nucleic acids when combined with immunochemical, histochemical or affinity detection systems. Two similar proteins, avidin and streptavidin, bind biotin very strongly and when coupled to fluorescent markers, enzymes or electron-dense proteins, can be exploited for the detection of nucleic acids. The use of fluorescent labeled antibodies raised against biotin can also be employed for detection. The biotin-labeled hybridization probe may be detected by use of a commercially available kit used for the detection of fluorescently labeled antibodies or by use of a transilluminator to detect the fluorescent group or protein.

Hybridization assays require the hybridization probe form stable Watson-Crick base pairs in order to localize the probe at a given sequence. The addition of biotin derivatives to the internucleotidic phosphorus



1 residues can result in some destabilization of the double  
stranded hybridization product. A series of biotin labeled  
probes can be prepared containing from one to approximately  
five biotin labels and the stability of the duplexes formed  
can be examined with biotin modified oligodeoxynucleotides in  
5 comparison with those unmodified. This can be accomplished  
by labeling of the oligodeoxynucleotides containing the  
correctly positioned (and number of) phosphorothioate  
diester(s) and isolation of the product using HPLC  
techniques. Duplex stability can be monitored by thermal  
10 denaturation experiments and circular dichroism spectra.

The ability of the biotin labeled oligodeoxy-  
nucleotide to function as a hybridization probe can then be  
examined using, for example, the 21-mer previously described.  
The sensitivity to detection of probes containing a varying  
15 number of biotin labels can be examined using commercially  
available fluorescent labeled proteins. "Spacing" the labels  
every two, three or more phosphorus residues can be the  
simplest route to enhance detection sensitivity.

In a second approach involving "post-assay"  
20 labeling, the phosphorothioate-containing probe is hybridized  
in one step; this avoids problems with the instability (if  
any) of the biotin labeled hybridization product.  
Subsequently, modification with the biotin label occurs, and  
after removal of the excess label, the protein solution is  
25 added for detection. This approach is conceptually similar  
to the one described for the visualization of DNA sequencing  
ladders and may also be the simplest approach to  
hybridization assays.

Hybridization experiments can also be performed  
30 with relatively long DNA fragments obtained from restriction  
digests and multiple phosphorothioate diesters can be

1 incorporated into such a fragment using DNA polymerase and  
nick-translation procedures. Radioisotopic labeling is  
accomplished by introducing "nicks" in the DNA with a dilute  
solution of DNase I and then elongating the nicked sites  
5 using DNA polymerase and the  $\alpha$ -[<sup>32</sup>P]dNTP substrates. The  
radioisotopic derivatives can then be replaced with the  
dNTP S derivatives and then hundreds of phosphorothioate  
diesters can be incorporated into the fragment. The simplest  
system to test hybridization can be one involving the M13 DNA  
10 being used in the sequencing reactions. For example, M13 RF  
(replicative form) DNA can be prepared in the conventional  
manner and then cleaved out a 444-mer to use as a  
hybridization probe. The 444-mer can then undergo  
nick-translation to incorporate the phosphorothioate diesters  
and then the modified and native sequences resolved by gel  
15 electrophoresis. A second sample of the M13 RF DNA, for  
example, can be digested such that the complementary 444-mer  
restriction fragment (in addition to others) is produced and  
transferred from an agarose gel to nitrocellulose or similar  
blotting membrane. The hybridization can then proceed  
20 followed by post-assay fluorescent labeling using, e.g.,  
monobromobimane; fluorescent labeling with hundreds of  
markers provides the desired detection sensitivity. Since  
the monobromobimane is largely non-fluorescent until it  
alkylates a sulfur containing functionality, the membrane  
25 background fluorescence is relatively low. The labeled  
marker can then be detected with relative ease.

In another embodiment of the present invention, DNA  
probes are generated from mRNA. Again, one can simply use  
the dNTP S derivatives, which function as substrates for  
30 reverse transcriptase, to form the complementary DNA strand  
for use as a hybridization probe. The use of the new

1 labeling approach provides well-characterized hybridization  
probes which can be used for the detection of specific DNA  
sequences, in the absence of radioisotopes, for example, in  
Southern blots, Northern blots, colony screening or plaque  
screening.

5 3. Specific Modification of Nucleic Acids with  
Fluorescent Markers or Spin Labels

In a further aspect of this invention, the labeling  
of specific phosphorothioate diesters is also valuable for  
structural studies involving fluorescent energy transfer  
10 techniques and electron spin resonance (ESR) techniques.

The application of these two spectroscopic  
techniques has long suffered from the difficulty in  
specifically attaching the desired probe to the nucleic acid  
fragment. The present procedure permits simple and rapid  
15 synthesis of a variety of nucleic acid sequences which can be  
easily modified with fluorescent markers or spin labels for  
spectroscopic studies.

Fluorescent Energy Transfer Techniques allow for a  
simple and rapid means for measurement of longer distances  
20 within the nucleic acid structure, complementing NMR  
techniques such as that of the Nuclear Overhauser Enhancement  
(NOE) which can only measure small distances in the nucleic  
acid.

The disadvantages of the energy transfer technique  
25 have previously been in the difficulty of easily placing the  
donor and acceptor chromophores in specific positions, and  
the questionable accuracy of the technique when the  
orientation of the chromophores is unknown.

These two shortfalls are eliminated by the labeling  
30 of specific phosphorothioate diesters pursuant to the  
methodology of the present invention. By controlling the

1 position of the phosphorothioate diester, the placement of a  
specific label becomes as rapid as it is simple. Since the  
label is oriented on the outer surface of the macromolecule a  
freely rotating chromophore is likely.

5 ESR spectra can be valuable for the study of  
biopolymer dynamics providing that the appropriate spin label  
can be specifically bound to the macromolecule of interest.  
In general, the technique has suffered a similar disadvantage  
to energy transfer experiments in the difficulty of  
specifically placing the label on the macromolecule. The use  
10 of the phosphorothioate diester can again be valuable in this  
respect. Nucleic acid fragments can be prepared with spin  
labels by exactly the same approach as described above for  
fluorescent markers. Specifically labeled probes can be  
designed and prepared for these ESR studies.

15 Other procedures which can be used in association  
with the instant technique involve optimization of  
fluorescence detection. These include, for example,  
1) altering the microenvironment of the labeled nucleic acid  
fragments in the gel matrix to increase the quantum yield of  
20 the fluorophore, 2) adjusting the excitation light energy to  
optimally fit the excitation spectrum of the dye and using  
filters to screen out all light energy (largely excitation  
wavelengths) other than the desired emission energy, and  
3) examining electronic detection as a means of automating  
25 the reading of the information present. The first two  
approaches together can be expected to increase the detection  
sensitivity by roughly one order of magnitude. Electronic  
methods can be expected to provide one or more additional  
orders of magnitude enhancement.

30 The following examples would assist in further  
detailing the subject invention herein.

EXAMPLES

1

1) Chemical Oligodeoxynucleotide Synthesis

5 Tp(s)T, the phosphorothioate diester derivative of TpT, is an example of the simplest phosphorothioate diester amenable to the labeling procedures described.

10 The (dT)<sub>15</sub> with phosphorothioate diesters 3' to thymidine residues 7, 8, and 9 were synthesized by using the phosphite triester methodology (Beaucage & Caruthers, Tetrahedron Lett., 22: 1859-1863, 1981) on a solid-phase CPG support. The synthesis was interrupted prior to the oxidation step when the incorporation of a phosphorothioate diester was desired. The normal oxidation step with 0.1 M I<sub>2</sub> in tetrahydrofuran/distilled water/lutidine (40:1:10) was replaced with a solution of 2.5 M sulfur in CS<sub>2</sub>/lutidine  
15 (1:1). The sulfur oxidation solution was injected directly onto the column with a syringe. After a reaction time of 1 h at ambient temperature, the column was washed with a 1:1 solution of CS, and lutidine to remove the residual sulfur. The column was then replaced on the machine, and the  
20 synthesis cycle was resumed. The 21-mer d(GCTATCGAAAGATCTCATAAG) was synthesized in an analogous manner. The synthesis was interrupted at every oxidation step to allow oxidation with the sulfur solution.

25 Both oligodeoxynucleotides were deprotected in ammonia at 50°C for 18 h. Isolation was done by reverse-phase HPLC on a 9.4 x 250 mm column of MOS-Hypersil using a buffer of 50mM triethylammonium acetate, pH 7.0 with a gradient of 20-65% acetonitrile in 40 min.

30

35

## 2) Solution Fluorescent Labeling Studies

1           The fluorophore of choice in this example,  
monobromobimane (MBB), was dissolved in acetonitrile, and  
stock solution (100mM) was stored in the dark at -20°C.

5           Typically, the oligodeoxynucleotides of interest  
were treated with an excess of monobromobimane, and the  
reaction was monitored by HPLC. Specifically, a solution of  
Tp(s)T (3.6 mM) in water was allowed to react overnight  
(18 h) with a 6-fold excess of monobromobimane (22 mM). The  
10           octamer (0.3 mM) in water was allowed to react with either a  
5-fold excess of MBB (1.5 mM) or a 10-fold excess of MBB  
(3.0 mM). The fragment Tp(s)Tp(s)Tp(s)T (0.43 mM, a  
phosphorothioate diester concentration of 1.29 mM) was  
treated with an 8-fold excess (with respect to the  
phosphorothioate diesters) of MBB (10.5 mM). Covalent  
15           fluorescent labeling of the 15-mer in solution (0.8 mM) with  
MBB was achieved at 7.5 mM MBB (3-fold excess for 2.4 mM  
phosphorothioate diester).

          The bimane-labeled Tp(s)T (see Figure 1) was  
isolated by reverse-phase HPLC on a 4.6 x 250 mm column of  
20           ODS-Hypersil with 50 mM triethylammonium acetate, pH 7.0, and  
a gradient of 0-70% acetonitrile in 1 h. The other labeling  
reactions were monitored by reverse-phase HPLC on a 4.5 x 250  
mm column of ODS-Hypersil with either 20 mM  $\text{KH}_2\text{PO}_4$ , pH 5.5,  
and a gradient of 0-70% methanol in 30 min (the octamer and  
25           tetramer) or 50 mM triethylammonium acetate, pH 7.0, and a  
gradient of 0-35% acetonitrile in 1 h (15-mer).

          Thin-layer chromatography studies were performed on  
silica gel thin-layer plates with a mobile phase of  
30           dichloromethane/methanol (9:1).

35

3) pH Stability Studies

1 Duplicate reaction mixtures of 6 nmol of  
bimane-labeled Tp(s)T were incubated at ambient temperature  
in 50 mM buffer at the appropriate pH values. The following  
5 buffers were used: pH values 3, 4 and 5, acetic  
acid/potassium acetate; pH values 6 and 7,  $K_2PO_4/K_2HPO_4$ ; pH  
values 8 and 9, Tris-HCl; pH values 10 and 11, CAPS. At  
various reaction times, the samples were analyzed by HPLC on  
a 4.6 x 250 mm column of ODS-Hypersil using 0.02 M potassium  
10 phosphate, pH 5.5, with a linear gradient of 0-70% methanol  
in 30 min. The bimane-labeled Tp(s)T eluted at 21 min, while  
the product TpT eluted at 16 min.

At low pH values (3-7) less than 5% of the triester  
was hydrolyzed after a 20 h incubation as determined by  
integration of the corresponding HPLC peaks. (see Figure 2).  
15 Upon incubation with Tris-HCl at pH 8 for 20 h, 11% of the  
triester was hydrolyzed. At pH 9, a 20 h incubation resulted  
in 40% of the hydrolysis product. The triester was  
completely hydrolyzed within 15 h at pH 10 and within 1 h at  
pH 11 (see Figure 2). HPLC analysis confirmed that  
20 hydrolysis occurred by cleavage of the P-S bond and formation  
of TpT as expected.

To further characterize the reaction of  
monobromobimane with a phosphorothioate diester, the reaction  
was performed with an oligodeoxynucleotide which at ambient  
25 temperature exists largely in the double-stranded form. The  
reaction of the octamer d[GpCp(s)CpCpGpGpGpC] with a 10-fold  
excess of monobromobimane was performed in either distilled  
water or Tris-HCl pH 7, at ambient temperature. The HPLC  
analysis after a 5-h incubation (Figure 3) showed the  
30 starting material (14.88 min), a monobromobimane hydrolysis  
product (15.3 min), a product peak (17.75 min), and

- monobromobimane (25.21 min). The starting material was completely consumed within 23 h. With a 5-fold excess of monobromobimane, the reaction was complete within 48 h. The reaction proceeded equally well with either the  $R_p$  or the  $S_p$  diastereoisomer. A control reaction containing an oligodeoxynucleotide with only phosphodiester failed to show any conversion to a labeled product.

4)  $^{31}\text{P}$  NMR Studies

- The  $^{31}\text{P}$  NMR studies were done at 121.5 MHz using a varian multinuclear FT-NMR. Positive chemical shift values are reported in parts per million (ppm) downfield from the external standard of aqueous 85% phosphoric acid. NMR analysis was done on a sample containing 1.2  $\mu\text{mol}$  of  $\text{Tp(s)Tp(s)Tp(s)T}$  (3.5  $\mu\text{mol}$  of phosphorothioate diesters) and 20 mM  $\text{Na}_2\text{EDTA}$ . The sample was adjusted to a volume of 250  $\mu\text{L}$  with  $\text{D}_2\text{O}$ . After NMR analysis of the tetramer, 10  $\mu\text{mol}$  of monobromobimane (a 3-fold excess with respect to the diesters) in 100  $\mu\text{L}$  of acetonitrile was added to the NMR tube with a final volume of 350  $\mu\text{L}$ . The sample was allowed to react for 2.5 h at ambient temperature in the dark. NMR analysis was then repeated.

5) Radioisotopic Labeling ( $^{32}\text{P}$  End Labeling)

- A reaction mixture in a final volume of 200  $\mu\text{L}$  containing 40.1  $\mu\text{M}$  15-mer (1  $A_{260}$  unit), 40.7  $\mu\text{M}$  ATP, 10 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol, 5  $\mu\text{g/mL}$  bovine serum albumin, 40 mM Tris-HCl, pH 8.7, 0.127  $\mu\text{M}$  (0.152 mCi) [ $\gamma$ - $^{32}\text{P}$ ]ATP, and 10 units of  $T_4$  polynucleotide kinase was incubated at 37°C for 18 h. After the addition of the reaction mixture to the Sep-pak cartridge (prewashed with 20 mL of methanol and 20 mL of distilled water), it was washed with 10 mL of 1% aqueous methanol to elute the unincorporated ATP and buffer salts. The oligodeoxynucleotide was eluted with 10 mL of 50% aqueous



1 methanol. The solution containing the DNA fragment was  
evaporated to dryness and redissolved in 0.4 M distilled  
water. Isolated yields ranged from 60 to 80%.

5 The 21-mer, 23.3  $\mu$ M (1  $A_{260}$  unit), was end labeled  
in an analogous manner but could not be eluted with aqueous  
methanol. In this case, the Sep-pak cartridge was prewashed  
with acetonitrile and distilled water. The unincorporated  
ATP and salts were then eluted with 1% aqueous acetonitrile  
while the oligodeoxynucleotide was eluted with 50% aqueous  
acetonitrile. Isolated yields also ranged from 60 to 80%.

10 6) Post-Assay Labeling

Gel electrophoresis was performed on 20 x 20 x  
0.04 cm or 34 x 42 x 0.04 cm gels of 20% acrylamide, 2%  
bis(acrylamide) [or 6% acrylamide and 0.6% bis(acrylamide)],  
50 mM  $Na_2EDTA$ , and 13 mM sodium persulfate. Post-assay  
15 labeling was performed both in the presence and in the  
absence of 7 M urea. The DNA was fixed in the gel by soaking  
it in 10% aqueous acetic acid for 5 min. The gel was then  
transferred to a 4 mM solution of monobromobimane in 50%  
aqueous acetonitrile and allowed to react overnight (18 h) in  
20 the dark. The gel was destained by shaking in 50% aqueous  
acetonitrile for 1 h. The short destaining appeared  
necessary because of minor reactions with the gel components  
and monobromobimane. Following a brief treatment (5 min) in  
60 or 75% aqueous dimethylformamide, the DNA was viewed on a  
25 standard long-wavelength ultraviolet transilluminator ( $\lambda_{max} =$   
366 nm). In some cases for internal standardization, the  
fluorescent bands of DNA were cut out of the gel and  
lyophilized before determination of the amount of DNA present  
in the gel via scintillation counting.

30

35

1       The effect of solvents on fluorescent intensity was  
also investigated. After post-assay labeling and destaining,  
the gels were treated with one of the following: 75% aqueous  
mixtures of methanol, ethanol, butanol, dimethylformamide, or  
concentrated glycerol. The gels were viewed using a long  
5   ultraviolet wavelength light transilluminator.

#### 7) Fluorescent Studies

      The fluorescence (excitation 385 nm, emission  
465 nm) of varying solutions of bimeane-labeled Tp(s)T in 5 mM  
KH<sub>2</sub>PO<sub>4</sub>, pH 4.5, was measured by using a fluorescence  
10   spectrophotometer, and a standard curve of fluorescence vs.  
phosphorothioate diester concentration was fitted to the data  
employing a linear least-squares analysis.

      After post-assay fluorescent labeling (see above)  
with monobromobimane, the 5'-<sup>32</sup>P end-labeled 15-mer was  
15   electroeluted for 2 h from a 20% polyacrylamide gel into  
dialysis tubing containing 0.5x TBE buffer. The solution was  
evaporated to dryness, redissolved in 1 mL of distilled  
water, and desalted using a column of Sephadex G-10. The DNA  
fragment was collected, evaporated to dryness, and  
20   redissolved in 3 mL of 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 4.5. The fluorescence  
of the solution was measured and the concentration of the  
15-mer determined by scintillation counting. The  
fluorescence as a function of concentration of the  
phosphorothioate diesters was plotted on the standard  
25   bimeane-labeled Tp(s)T curve.

      In similar fashion, the 5'-<sup>32</sup>P end-labeled 21-mer  
was electroeluted for 24 h from the polyacrylamide gel after  
post-assay labeling. The solution was evaporated to dryness  
and redissolved in 0.5 mL of distilled water. In this case,  
30   the solution containing the 21-mer was adjusted to 10 mM  
MgCl<sub>2</sub> and 2 M ammonium acetate, 1 volume of ice-cold

1 acetonitrile was added, and the solution was kept at  $-70^{\circ}\text{C}$   
for 18 h. The salt precipitated out of solution while  
essentially all of the DNA remained in the supernatant. The  
solubility of the labeled 21-mer in acetonitrile is largely a  
5 result of the increased hydrophobicity conferred upon the  
oligonucleotide due to the presence of the bimane residues.  
The supernatant was decanted, evaporated to dryness, and  
dissolved in 3 mL of 5 mM  $\text{KH}_2\text{PO}_4$ , pH 4.5. The fluorescence  
and radioactivity were measured and compared with the  
standard curve.

10 8) DNA Polymerase and Restriction Endonuclease Reactions

M13 mp18 DNA was converted to the replicative form  
(RF) as follows. The template DNA (2.5 ug) and universal  
primer (0.1 ug) were annealed in 25 uL of buffer containing  
100 mM NaCl, 20 mM  $\text{MgCl}_2$ , and 100 mM Tris-HCl, pH 8.0, by  
15 heating the mixture to  $56^{\circ}\text{C}$  for 15 min followed by slow  
cooling to ambient temperature. The final 50-uL reaction  
mixture containing dATP, dGTP, dCTP, dTTP (500 uM each), ATP  
(1 mM), DNA polymerase 1 (Escherichia coli, 10 units), and  
T4 DNA ligase (8 units) was incubated overnight at  $16^{\circ}\text{C}$ .  
20 Substitution of the appropriate dNTP  $\alpha$ S derivative(s) for the  
corresponding dNTP(s) essentially as described (Taylor,  
et al., Nucleic Acids Res., 13: 8749-8764, 1985) allowed the  
enzymatic incorporation of phosphorothioate diesters in place  
of phosphodiester. In some cases for internal  
25 standardization,  $\alpha$ -[ $^{35}\text{S}$ ]-dATP (1.15 Ci/mmol) was employed in  
the elongation reaction.

Restriction digests with AvaI and HpaII were  
performed as follows. The AvaI reaction mixture contained RF  
M13mp19 DNA, 100 mM NaCl, 20 mM  $\text{MgCl}_2$ , and 100 mM Tris-HCl,  
30 pH 8.0. The HpaII reaction mixture contained RF M13mp18 DNA,  
3 mM KCl, 5 mM  $\text{MgCl}_2$ , 100 ug/mL BSA, and 5 mM Tris-HCl,

1 pH 7.4. The reactions were initiated by the addition of the  
enzyme and incubated at 37°C for 2 h. The reaction mixture  
was loaded onto 6% acrylamide, 0.6% bis(acrylamide) gels (20  
x 20 x 0.04 cm or 34 x 42 x 0.04 cm) containing 3 mM Na<sub>2</sub>EDTA,  
7 M urea, and 50 mM Tris-borate, pH 8.3. Fluorescent  
5 labeling proceeded as described above.

9) Detection of Nucleic Acids

The 5'-<sup>32</sup>P end-labeled 21-mer was viewed on a  
transilluminator (  $\lambda_{\text{max}}$  = 366 nm) after gel analysis and  
post-assay labeling. The bluish green bands were excised  
10 from the gel and lyophilized, and the amount of DNA present  
was determined by scintillation counting. The amount of the  
oligodeoxynucleotide visible as a result of the bimane  
fluorescence has decreased such that 500 fmol (500 x 10<sup>-15</sup>  
mol) of the DNA fragments could be observed.

15 Longer DNA fragments containing phosphorothioates  
can be prepared by enzymatic synthesis if the dNTP substrates  
are substituted by the  $\alpha$ -thio derivatives (Taylor et al.,  
Nucleic Acids Res., 13: 8749-8764, 1985). In order to  
generate fragments of defined length, an oligonucleotide  
20 primer was extended using an M13mp18 or M13mp19 template and  
the resulting material was hydrolyzed with a restriction  
endonuclease. It was possible to prepare M13 RF DNA  
containing phosphorothioates at each position. Cleavage of  
the elongated DNA with HpaII produced fragments which  
25 migrated in the 6% polyacrylamide gel and could be visualized  
by post-assay fluorescent labeling (Figure 4A). A similar  
experiment with the AvaI restriction endonuclease produced a  
444-nucleotide fragment which could be visualized by  
post-assay covalent labeling (Figure 4B). Some high  
30 molecular weight DNA could also be observed in this gel at  
the edge of the sample well (Figure 4B). With the 444-mer,

1 the bands were excised, and the amount of DNA was determined  
by scintillation counting. Approximately 40 fmol ( $40 \times 10^{-15}$   
mol) of the 444-mer (containing a maximum of 104  
bimane-labeled phosphorothioate diesters) could be visualized  
in this experiment.

5 10) Synthesis of oligodeoxynucleotides containing  
a single phosphorothioate diester

Two oligonucleotides were synthesized for covalent  
attachment of a variety of reporter groups, including spin  
labels, fluorophores and drug derivatives. A  
10 dodecadeoxynucleotide and an eicosodeoxynucleotide were  
chemically synthesized by the phosphoramidite method  
described in Example 1 and altering the oxidation step at the  
appropriate cycle, resulting in two phosphorus diastereomers  
(Rp and Sp). It is possible to prepare the oligonucleotide  
15 such that it contains a pure phosphorus diastereoisomer as  
described [Connolly et al., Biochemistry 23: 3443-3453,  
1984; Taylor et al., 1985].

Specifically, the dodecamer has the sequence  
d[CGCA(s)AAAAAGCG] and the eicosomer has the sequence  
20 d[CGTACTAGTT(s)AACTAGTACG].

Additionally Tp(s)T was reacted with a number of  
fluorophores or reporter groups containing a variety of  
functional groups. Three functionalities,  $\gamma$ -bromo- $\alpha, \beta$ -un-  
saturated carbonyls, iodo (or bromo) acetamides, and  
25 aziridinyl sulfonamides, were observed to effectively label  
phosphorothioate diesters and produce the corresponding  
phosphorothioate triester carrying the desired reporter  
group.

30

35

11) Phosphorothioate triester1 oligodeoxynucleotides carrying various reporter groups

Oligodeoxynucleotides of Example 10 containing a single covalently bound reporter group (Fig. 5) were obtained by incubation of the phosphorothioate-containing DNA fragment with the reporter group of choice in aqueous or largely aqueous solutions at pH values from 5 to 8. These reactions were performed at 25 to 50°C and usually proceeded with yields greater than 85% after 24 h at 50°C. Resolution of the reaction mixture and isolation of the triester product was accomplished by using HPLC (4.6 X 250 mm Hypersil-ODS with 0.02 M  $\text{KH}_2\text{PO}_4$  pH 5.5 and a methanol gradient). Modification of the phosphorothioate was observed to be more efficient for the single-stranded dodecamer than the self-complementary eicosomer. This difference in reactivity was partially overcome when the reaction mixture was heated at 50°C. In the absence of the phosphorothioate diester, control reactions using native oligodeoxynucleotides did not result in any significant labeling.

## a) Attachment of a PROXYL spin label:

20 The reaction to produce the compound in Fig. 5a was conducted as described above using the following specific conditions: 10 mM 3-(2-iodoacetamido)PROXYL, 0.15 mM dodecamer, pH 8.0 (phosphate) at 50°C in a solution containing 4% DMP. Similar conditions were employed to label the eicosomer.

## b) Attachment of a CC-1065 drug analogue: a derivative of the dihydropyrroloindole subunit:

30 The reaction to produce the compound in Fig. 5b was conducted as described above using the following specific conditions: 5 mM dihydropyrroloindole derivative,

1 0.07 mM dodecamer, pH 8.0 (Tris) at 50°C in a solution  
containing 60% DMF. This reaction required 48 h at 50°C or  
80 h at 25°C at which time it was 70-80% complete. Similar  
conditions were employed to label the eicosomer.

5 c) Attachment of a sulfonamide-linked dansyl  
fluorophore:

The reaction to produce the compound in Fig.  
5c was conducted as described above using the following  
specific conditions: 12 mM N-dansylaziridine, 0.34 mM  
dodecamer, pH 8.0 (phosphate) at 25°C in a solution  
10 containing 50% acetonitrile. Similar conditions were  
employed to label the eicosomer.

At 50°C, HPLC analysis of the dansylaziridine  
reaction indicated the presence of minor products, suggesting  
some nonspecific reaction with the DNA. Labeling conducted  
15 at 25°C (pH 8.0) proceeded more slowly, but did not indicate  
the presence of any species other than the desired product  
and starting materials. However, the possibility of some  
nonspecific modification of the DNA even at 25°C can not be  
excluded.

20 d) Attachment of an N-linked dansyl fluorophore:

The reaction to produce the compound in Fig.  
5d was conducted as described above using the following  
specific conditions: 10 mM 1,5-I-AEDANS, 0.80 mM dodecamer,  
pH 6.0 (phosphate) at 50°C in a solution containing 25% DMF.  
25 Similar conditions were employed to label the eicosomer.

12) Stability and properties of phosphorothioate  
triesters from examples 10 and 11

The unlabeled dodecamer helix, d[CGCA(s)AAAAAGCG]  
d[CGCTTTTTTGCG], exhibited a  $T_m$  of 55°C, and this was  
30 indistinguishable from the  $T_m$  values obtained for the PROXYL-  
labeled (a in Figure 5) or drug-labeled (b in Figure 5)

1 helices. The  $T_m$  value for the self-complementary eicosomer,  
d[CGTACTAGTT(s)AACTAGTACG]<sub>2</sub> with two labels was also largely  
unchanged (68.5°C) in comparison to the unlabeled fragment  
( $T_m$  = 67°C).

5 The hydrolytic stability of the phosphorothioate  
triesters is an important practical consideration for the  
value of such derivatives in many studies. Hydrolysis of the  
triesters proceeded by desulfurization (monitored by HPLC and  
confirmed by comparison with authentic standards). No  
10 detectable cleavage of the oligodeoxynucleotide at the point  
of attachment was observed. This agrees with the results of  
ethylated or hydroxyethylated derivatives, which result in  
primarily desulfurization and only very minor amounts of  
chain cleavage.

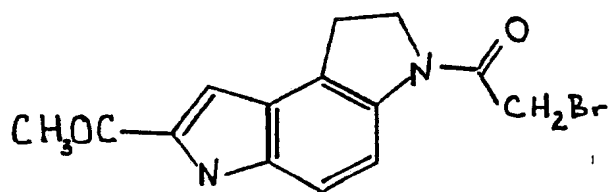
15 Less than 5% of the Tp(s)T triester carrying the  
PROXYL spin label was hydrolyzed after 24 h at pH 7. At pH 8  
this increased to 28%, and at pH 10 the triester was  
completely hydrolyzed within 11 h. With longer fragments,  
the hydrolytic stability of the triester increased [the  
20 labeled dodecamer was hydrolyzed <1%, 30%, and 99% at pH  
values 7, 8, and 10, respectively; the values for the  
eicosomer were <1%, 2%, and 63% (24 h)]. The triester  
prepared from a  $\gamma$ -bromo- $\alpha$ ,  $\beta$ -unsaturated carbonyl (b in  
Figure 5) exhibited stability similar to that of the  
PROXYL-labeled derivatives while that resulting from reaction  
25 with the aziridinyl sulfonamide (c in Figure 5) was more  
stable [the Tp(s)T-labeled triester was hydrolyzed <1% (pH  
7), 5% (pH 8), and 34% (pH 10) after 24 h at ambient  
temperature].

30 It is noteworthy that the triester produced from  
1,5-I-AEDANS and Tp(s)T was significantly less stable than  
the PROXYL-labeled derivative although the triesters formed



1 both resulted from iodoacetamides. The AEDANS-labeled dimer  
exhibited 19% (pH 7) and 88% (pH 8) hydrolysis (24 h); it was  
completely hydrolyzed within 2 h at pH 10. However, the  
AEDANS-labeled dodecamer (d in Figure 5) exhibited only  
1%, 49%, and 99% hydrolysis at the same respective pH values  
5 (24 h).

An additional dodecamer was labeled with the  
bromoacetamideo derivative i. Although the three  
acetamido-linked adducts are similar in structure, that  
prepared from i proved to be more stable than either a or d  
10 (Figure 1) (only 13% of the triester formed from i was  
hydrolyzed after 24 h at pH 8.0).  
Derivative i:



WE CLAIM:

- 1           1. A nucleic acid detection product comprising a phosphorothioate diester covalently complexed with an internucleotidic residue wherein said phosphorothioate diester is also complexed with a detectable marker.
- 5           2. The product of Claim 1 wherein said marker is a fluorescent marker.
3. The product of Claim 2 wherein said fluorescent marker is monobromobimane.
4. The product of Claim 2 wherein said fluorescent  
10       marker is bromomethylcoumarin.
5. The product of Claim 2 wherein said fluorescent marker carries a bromoacetamide, iodoacetamide, an aziridinosulfonamide, or a  $\gamma$ -bromo- $\alpha,\beta$ -unsaturated carbonyl group.
- 15          6. The product of Claim 1 wherein said marker is biotin, or a biotin derivative.
7. The product of Claim 1 wherein said marker is a spin label or spin probe.
8. The product of Claim 7 wherein said spin label  
20       is PROXYL.
9. The product of Claim 1 wherein said marker is a metal complex.
10. The product of Claim 1 wherein said marker is a drug or a drug analog.
- 25          11. The product of Claim 10 wherein said drug analog is a dihydropyrroloindole subunit of CC-1065.
12. A method of labeling nucleic acids comprising reacting a nucleic acid having a phosphorothioate diester in at least one internucleotidic phosphorus residue, with a  
30       marker to form an internucleotidic residue-phosphorothioate diester-marker complex.

13. A method of detecting nucleic acids, comprising  
1 introducing at least one phosphorothioate diester into a DNA  
fragment or oligodeoxynucleotide, labeling said  
phosphorothioate diester with a marker, and detecting the  
marker in complex with the nucleic acid.

5 14. A method of identifying nucleotides comprising  
site-selectively introducing at least one phosphorothioate  
diester into at least one internucleotidic residue of a DNA  
fragment or oligodeoxynucleotide, labeling said  
phosphorothioate diester with a detectable marker, and  
10 detecting said marker.

15. The method of Claim 12, 13 or 14 wherein said  
marker is a fluorescent marker.

16. The method of Claim 15 wherein said fluorescent  
marker is monobromobimane.

15 17. The method of Claim 15 wherein said fluorescent  
marker is bromomethylcoumarin.

18. The method of Claim 15 wherein said fluorescent  
marker carries a bromoacetamide, iodoacetamide, an  
aziridinosulfonamide, or a  $\gamma$ -bromo- $\alpha,\beta$ -unsaturated  
20 carbonyl group.

19. The method of Claim 12, 13 or 14 wherein said  
marker is biotin or a biotin derivative.

20. The method of Claim 12, 13 or 14 wherein said  
marker is a spin label or spin probe.

25 21. The method of Claim 20 wherein said spin label  
is PROXYL.

22. The method of Claim 13 or 14 wherein said  
marker is a metal complex.

23. The method of Claim 12 wherein said marker is a  
30 drug or a drug analog.

1           24. The method of Claim 13 or 14, wherein said  
labeling is conducted subsequent to resolution of the nucleic  
acid by performance of a biochemical assay.

5           25. The method of Claim 13 or 14, wherein said  
labeling is conducted prior to resolution of the nucleic acid  
by a biochemical assay.

26. The method of Claim 24 or 25, wherein said  
biochemical assay is gel electrophoresis.

10          27. The method of Claim 12, 13 or 14 wherein said  
phosphorothioate diester is selectively introduced into a DNA  
fragment or oligodeoxynucleotide at a specific nucleotidyl  
site.

15          28. The method of Claim 27 wherein said  
phosphorothioate diester is selectively introduced into said  
DNA fragment or said oligodeoxynucleotide by an oxidation  
reaction in the presence of elemental sulfur, CS<sub>2</sub> and  
lutidine; and subsequently by a hydrolysis reaction in the  
presence of a base.

20          29. The method of Claim 27 wherein said  
phosphorothioate diester is introduced into said DNA  
fragment or said oligodeoxynucleotide by chemical or  
enzymatic techniques.

25          30. A method of DNA sequencing comprising:  
a. introducing at least one phosphorothioate  
diester into at least one selected site of a  
DNA fragment or oligodeoxynucleotide by  
enzymatic dideoxy sequencing procedures;  
b. labeling each of said phosphorothioate diester  
with a marker; and  
30          c. detecting said DNA sequence.

35

1           31. The method of Claim 30 which further comprises  
generating said DNA fragment or oligodeoxynucleotide from  
dNTP  $\alpha$ S derivatives and at least one of dideoxy derivative  
(ddNTP).

5           32. The method of Claim 30, wherein said detection  
procedure is automated.

33. A method of DNA hybridization comprising:  
a. generating DNA fragments or  
oligodeoxynucleotides of reproducible size by  
selective chemical means;  
10       b. resolving said DNA fragments or  
oligodeoxynucleotides by a biochemical assay;  
c. hybridizing said DNA fragments or  
oligodeoxynucleotides to a DNA hybridization  
probe having at least one internucleotidic  
15       phosphorothioate diester;  
d. labeling said DNA hybridization probe with at  
least one detectable marker after said  
hybridization;  
e. detecting at least one marker in complex with  
20       said hybridized probe.

34. A method of DNA hybridization comprising:  
a. generating DNA fragments or  
oligodeoxynucleotides of reproducible size by  
selective chemical means;  
25       b. resolving said DNA fragments or  
oligodeoxynucleotides by a biochemical assay;  
c. labeling a DNA hybridization probe having at  
least one internucleotidic phosphorothiate  
diester with at least one detectable marker  
30       before hybridization with said DNA fragments or  
oligodeoxynucleotides;

- 1                   d. hybridizing said DNA fragments or  
                  oligodeoxynucleotides to said labeled DNA  
                  hybridization probe;
- e. detecting at least one marker in complex with  
                  said hybridized probe.
- 5                   35. The method of Claim 33 or 34 wherein said DNA  
                  hybridization probe has an internucleotidic phosphorothioate  
                  diester at each internucleotidic phosphorus.
36. The method of Claim 33 or 34 wherein said  
10                  selective chemical means is digestion with restriction  
                  endonucleases.
37. The method of Claim 33 or 34 wherein said  
                  biochemical assay for resolution of said DNA fragments or  
                  oligodeoxynucleotides is polyacrylamide or agarose gel  
                  electrophoresis.
- 15                  38. The method of Claim 33 or 34 wherein said DNA  
                  hybridization probe is prepared by the steps comprising:
- a. obtaining DNA fragments from restriction  
                  digests; and
- b. incorporating more than one phosphorothioate  
20                  diester into said DNA fragments by DNA  
                  polymerase and nick translation procedures.
39. The method of Claim 33 or 34 wherein said DNA  
                  hybridization probe is prepared from mRNA.
40. The method of Claim 39 wherein said  
25                  hybridization probe is prepared from mRNA by the steps  
                  comprising:
- a. providing dNTP  $\alpha$ S derivatives to  
                  function as substrates for reverse  
                  transcriptase; and
- 30                  b. synthesizing a complementary DNA strand to said  
                  mRNA by the action of reverse transcriptase,  
                  thereby forming said hybridization DNA probe.
- 35

41. The method of DNA hybridization according to  
1 Claim 33 or 34 wherein said marker is a fluorescent marker.

42. The method of DNA hybridization according to  
Claim 33 or 34 wherein said marker is monobromobimane.

43. The method of DNA hybridization according to  
5 Claim 33 or 34 wherein said marker is bromomethylcoumarin.

44. The method of DNA hybridization according to  
Claim 33 or 34 wherein said marker carries a bromoacetamide,  
iodoacetamide, aziridinosulfonamide or  $\gamma$ -bromo- $\alpha, \beta$ -  
unsaturated carbonyl group.

45. The method of DNA hybridization according to  
10 Claim 33 or 34 wherein said marker is biotin or a biotin  
derivative, and wherein the resulting product is a  
biotin-labeled hybridization probe.

46. The method of Claim 45 wherein said detection  
15 comprises:

- a. attaching a detectable protein to said  
biotin-labeled hybridization probe; and
- b. identifying said probe.

47. The method of DNA hybridization according to  
20 Claim 46 wherein said protein is avidin or streptavidin.

48. The method of DNA hybridization according to  
Claim 46 wherein said protein is an antibody to said  
biotin-labeled hybridization probe.

49. The method according to Claim 33 or 34 wherein  
25 said DNA fragment or oligodeoxynucleotide is transferred to a  
blotting membrane for the detection of specific DNA sequences  
by Southern blots, Northern blots, colony screening or plaque  
screening, thereby identifying DNA sequences under  
investigation.

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50. A method for DNA detection comprising:

- a. generating a DNA probe from DNA fragments or oligodeoxynucleotides having at least one phosphorothioate diester;
- b. hybridizing said DNA probe to a selected DNA sequence under investigation;
- c. labeling said probe with at least one detectable marker subsequent to said hybridization and thereby introducing said marker into the phosphorothioate diester; and
- d. locating said selected DNA sequences under investigation by detecting said marker complexed with said hybridization probe.

51. A method for identifying DNA sequences comprising:

- a. generating a DNA probe from DNA fragments or oligodeoxynucleotides having at least one phosphorothioate diester;
- b. hybridizing said DNA probe to a selected DNA sequence under investigation;
- c. labeling said probe with at least one detectable marker subsequent to said hybridization and thereby introducing said marker into the phosphorothioate diester;
- d. locating said selected DNA sequences under investigation by detecting said marker complexed with said hybridization probe; and
- e. directing drug delivery to said DNA sequence.

52. The method of Claim 51 which further comprises activating said DNA sequence.

30            53. The method of Claim 51 which further comprises  
inactivating said DNA sequence.

35



1        54. The method of Claim 51 which further comprises  
degrading said DNA sequence.

55. A method of targeting a nucleic acid for  
sequence-specific drug delivery which comprises:

- 5        a. preparing a sequence-specific nucleic acid  
probe having at least one phosphorothioate  
diester;
- 10       b. labeling said phosphorothioate diester of said  
probe with a drug or a drug analogue; and
- 10       c. hybridizing said probe with said nucleic acid  
and thereby delivering said drug to a specific nucleic acid  
target.

56. The method of Claim 55 wherein step b is  
performed subsequent to step c.

15       57. The method of Claim 55 wherein said drug  
analogue is a dihydropyrroloindole subunit of CC-1065.

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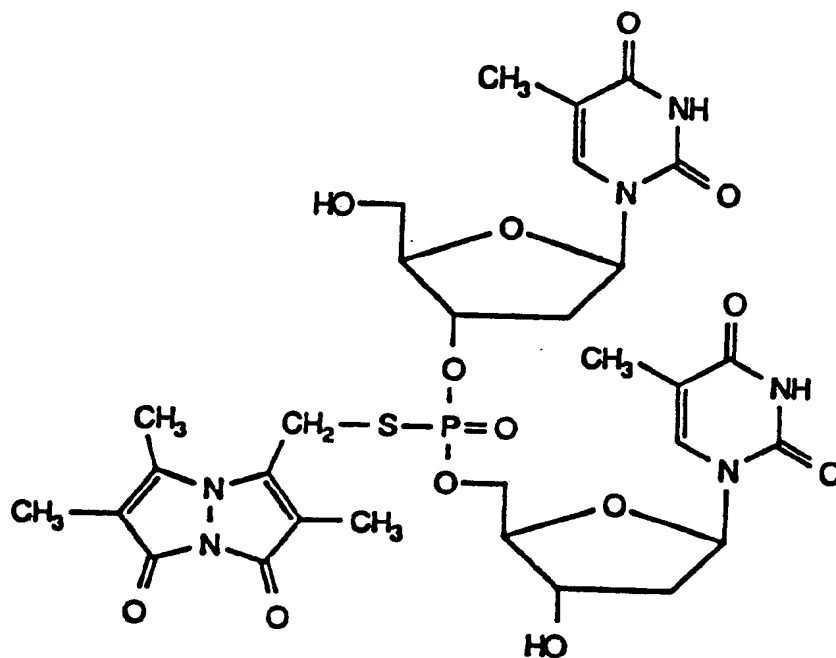


FIG. 1

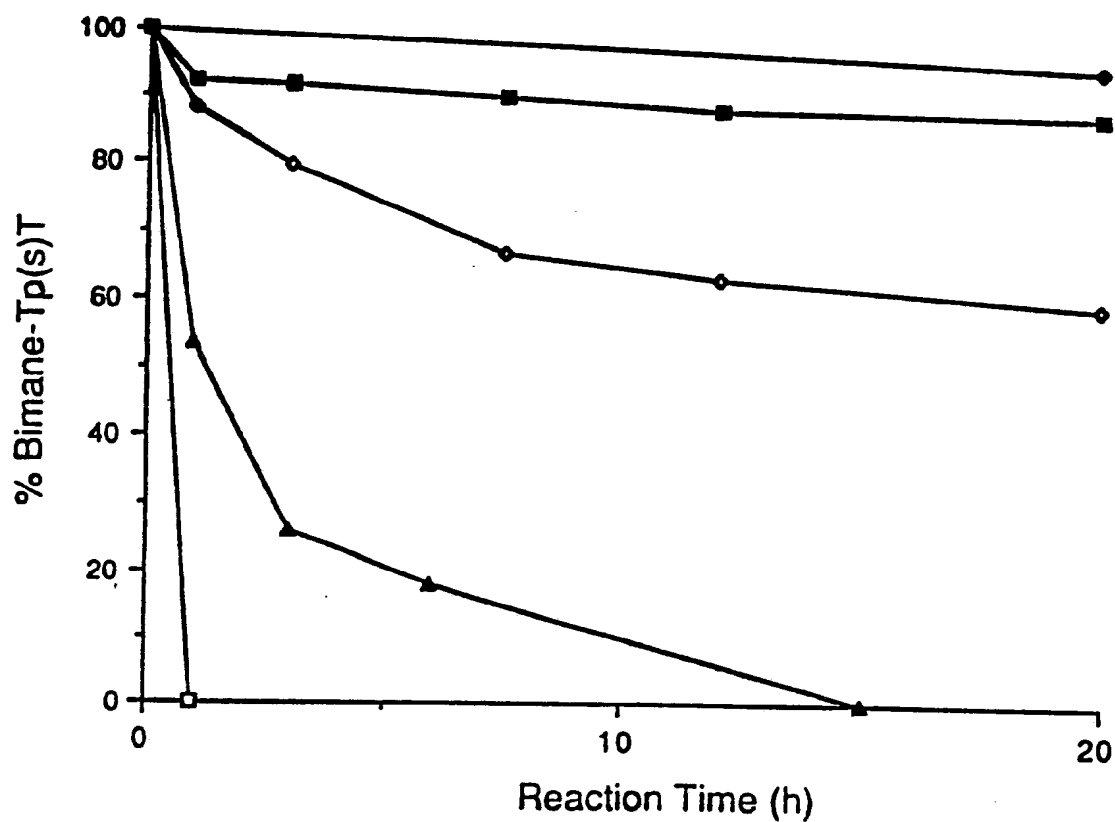


FIG. 2

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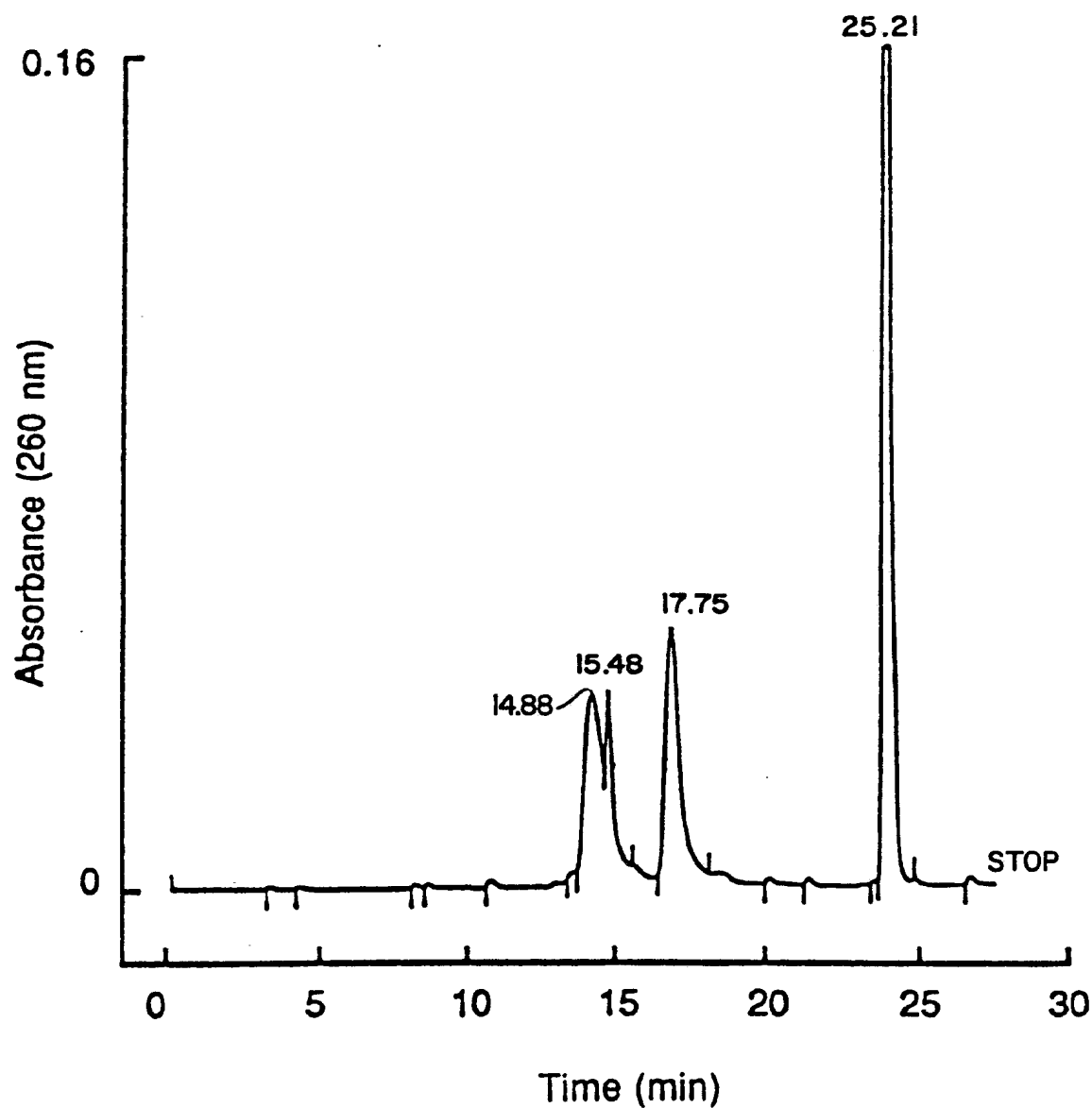


FIG. 3

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FIG.4A



**SUBSTITUTE SHEET**

FIG. 4B

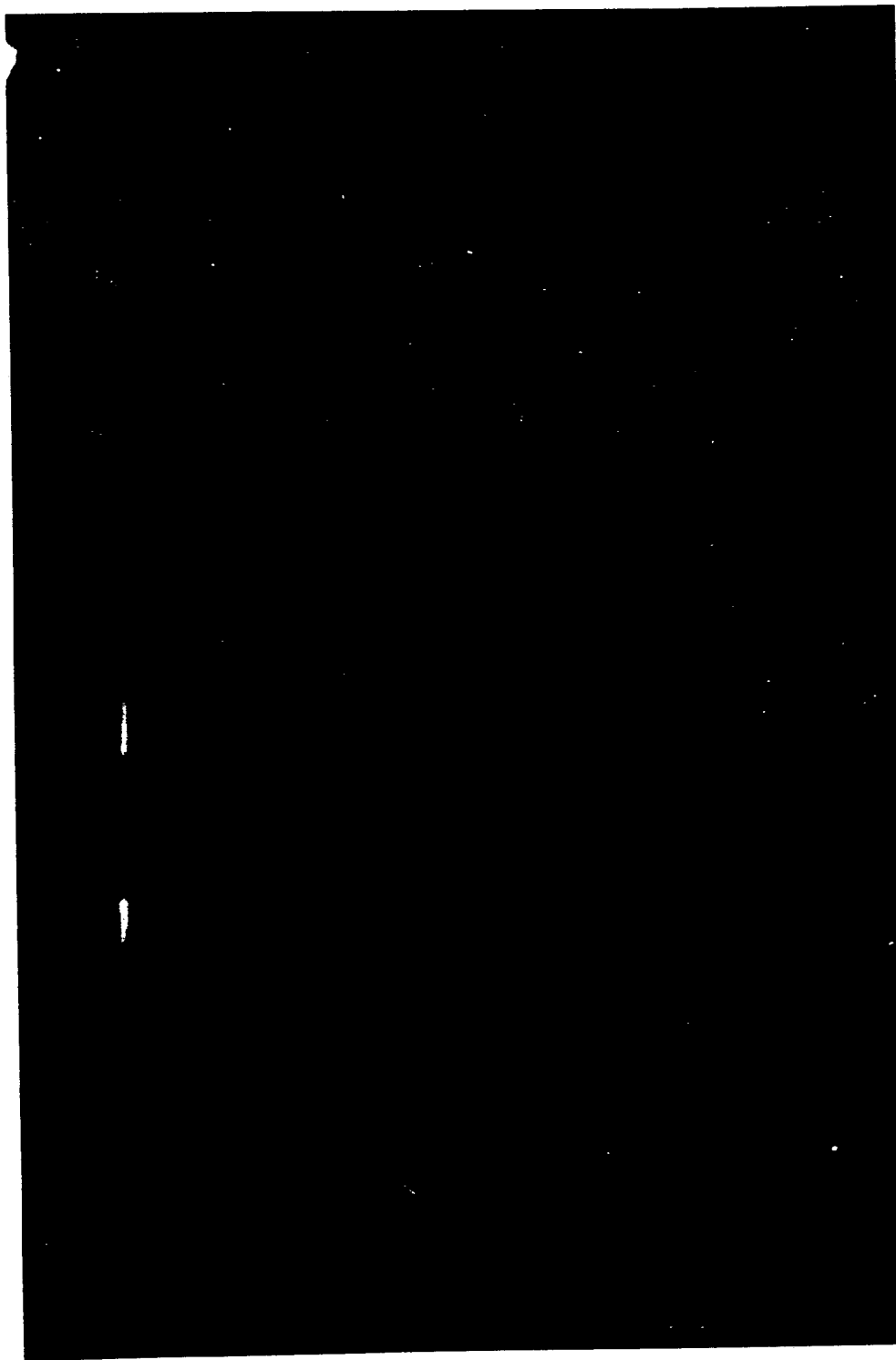
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**SUBSTITUTE SHEET**

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FIG. 5a

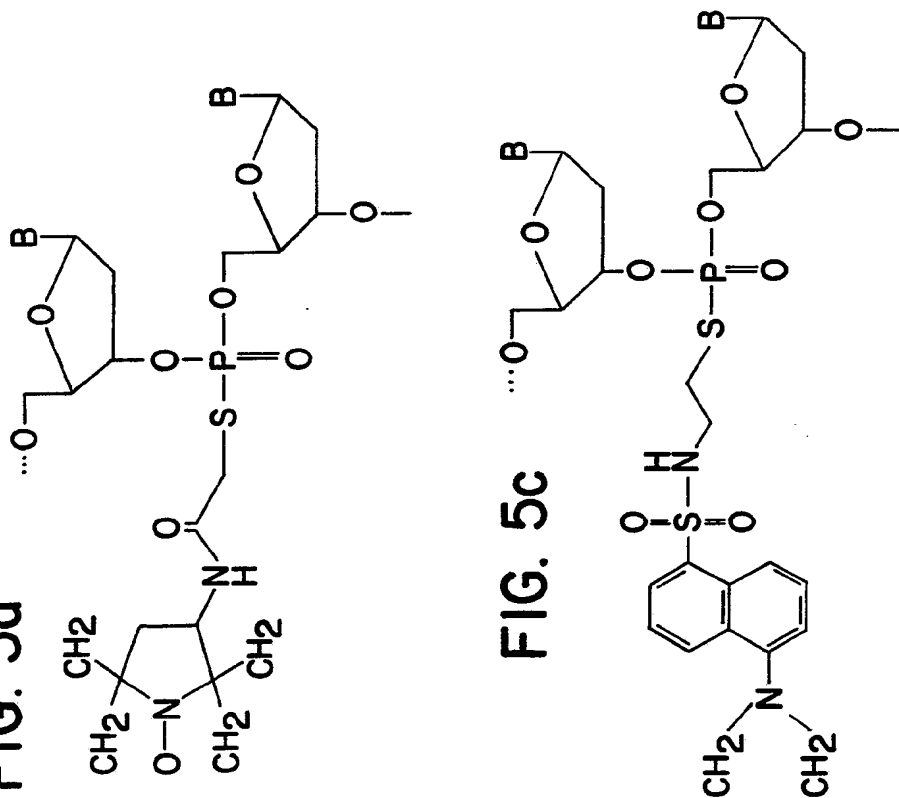


FIG. 5b

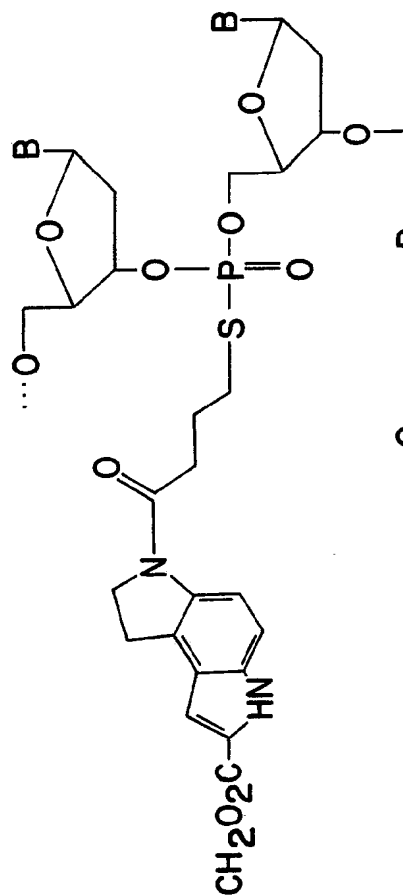


FIG. 5c

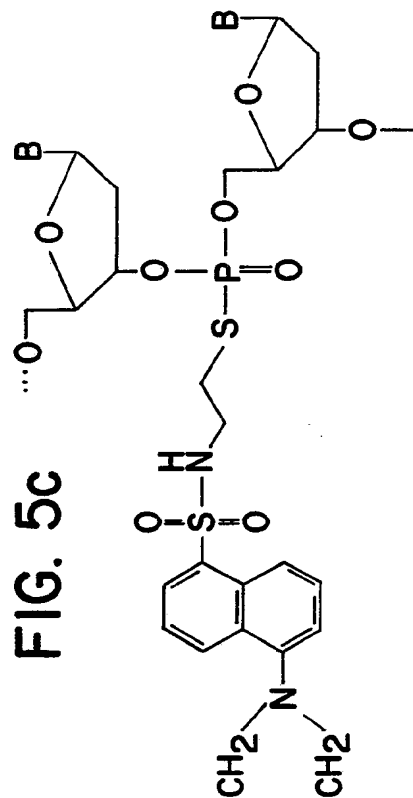
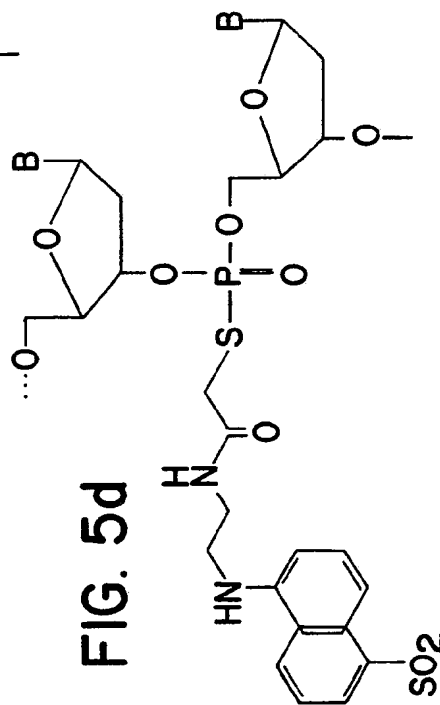


FIG. 5d



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00182

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/68 U.S. CL.: 435/6; 536/27						
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">U.S. CL.</td> <td style="padding: 5px;">435/6, 436/800, 803, 804, 536/27, 935/86</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div> <p style="margin-top: 10px;">Dialog Data Base: Biotech, APS</p>			Classification System	Classification Symbols	U.S. CL.	435/6, 436/800, 803, 804, 536/27, 935/86
Classification System	Classification Symbols					
U.S. CL.	435/6, 436/800, 803, 804, 536/27, 935/86					
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>						
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>				
Y	US, A, 4,358,535, (FALKOW ET AL.) 09 November 1982 (See columns 4 and 5).	1-29 & 50				
Y,E	US, A, 4,910,300, (URDEA ET AL.) 20 March 1990 (See examples 10-13 and the claims).	1-29 & 50				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>						
<b>IV. CERTIFICATION</b>						
Date of the Actual Completion of the International Search  02 MAY 1990	Date of Mailing of this International Search Report  <div style="text-align: center; font-size: 1.2em;">11 JUN 1990</div>					
International Searching Authority  ISA/US	Signature of Authorized Officer  AMELIA BURGESS YARBROUGH					